Vol. XXXII. *January, 1917.* *No. 1.*

BIOLOGICAL BULLETIN

CYTOPLASMIC STRUCTURES IN THE MALE GERM CELLS OF RHOMALEUM MICROPTERUM BEAUV.

HAROLD H. PLOUGH,

ZOÖLOGICAL LABORATORY, COLUMBIA UNIVERSITY.

In a recent paper Lewis and Robertson gave an account of certain cytoplasmic structures in the male germ cells of *Chorthippus curtipennis* Scud. as seen by the tissue culture method. It is the purpose of this paper to give somewhat similar data for the Florida lubber grasshopper, *Rhomaleum micropterum* Beauv., on the basis of both fixed and living material. My observations were made mainly in order to determine the origin and significance of the chromatoid body. They closely parallel those of the previous authors, and may perhaps clear up some doubtful points.

While studying the maturation phenomena in *Rhomaleum* in 1914 it was found that a small round densely staining body was always present in the cytoplasm of the cells of the late growth period. Further study disclosed the fact that this body appeared in the very early growth period, enlarged up to the stage of diakinesis, and at both the first and second maturation divisions passed unchanged into one of the two daughter cells. During the metamorphosis of the spermatid this body passed gradually down into the tail region of the future spermatozoon, and was eventually cast off and degenerated in the lower end of the follicles. This history is exactly parallel to that of a similar but larger body described by Dr. E. B. Wilson ('13) in the spermatogenesis of the hemipter *Pentatoma senilis*, and called by him the "chromatoid body." It is now known that a granule or granules of similar behavior are present in the developing male germ cells of the following forms: horse, pig, bull, rabbit, crayfish and

probably in the rat, mouse, and a number of Hempitera. It seems probable therefore that the appearance and elimination of such material from the spermatozoa is a fairly general phenomenon throughout the animal kingdom.

The recent study of Lewis and Robertson on the living germ cells of *Chorthippus curtipennis* showed that certain granules were present in the spermatogonia and passed on somewhat enlarged into the metamorphosing spermatids. From the fact that these granules stained with neutral red in contrast to the mitochondria they were called "neutral red granules." Up to the time their paper was published the work with *Rhomaleum* had been carried exclusively with fixed material, but in view of the similarity of behavior between these granules and the chromatoid body in the Florida grasshopper it was believed that they were of similar material and significance. Work on the living male germ cells of *Rhomaleum* during the past summer has confirmed this belief in every respect, and has made it possible to add a few facts to the data of the previous authors.

The material from which the observations were made was secured from the Supply Department at Woods Hole during the summers of 1915 and 1916. That used for permanent preparations was fixed in the usual fixatives including the modified Flemming's fluid used for the Benda mitochondrial technique. A large number of different staining methods were employed including the Auerbach mixture, the Borel stain, together with the Altmann acid fuchsin stain and the alizarin-crystal violet combination of Benda for mitochondria. The osmic mixtures were invariably the best cytoplasmic fixatives and from material so treated all of the drawings from permanent preparations were made. The methods used with the living material were for the most part those of Lewis and Robertson. Intravitam staining with janus green B and neutral red was employed, the stains being used both separately and together. The culture medium used was the modified Locke's solution of these authors made up with sea water, though some follicles were stained in Ringer's solution with fair success. The tissue cultures were only partially successful, most of the cells being abnormal after thirty-six hours. This was probably due to lack of familiarity with the

technique. Ample data for the purposes of this paper were secured, however, since cells can be secured at any desired stage without watching any particular cell for any great length of time.

Observations.

However we may interpret the behavior of the chromosomes, *Rhomaleum* corresponds in all essentials with the account of orthopteran spermatogenesis given by Davis ('08) for *Dissosteira*, with the *Hippiscus* type of the Acrididæ as described by McClung ('14) and with *Phrynotettix* (Wenrich, '16). A connected account of the stages through which the chromosomes of *Rhomaleum* pass during the growth period would therefore correspond in most respects with that given by Davis.

In the spermatogonia the cytoplasm in fixed material is comparatively clear except for a minute fibrillar network. In addition several minute but darkly staining granules can usually be seen scattered about the cytoplasm. Mitochondria are apparently never visible in the spermatogonia even when the material is fixed in the modified Flemming and stained with the so-called mitochondrial stains. In living material however the cytoplasm shows no fibrillar network, but the mitochondria appear irregularly scattered about the cytoplasm or more often in a fairly dense mass close to the nuclear wall. In the resting stages in *Rhomaleum* they are very delicate granules, staining brilliantly with janus green, and show very little tendency toward any thread-like arrangement. No dividing primary spermatogonial cell was observed. In the resting stages of the secondary spermatogonia the mitochondria apparently have a tendency to be less scattered and more densely crowded next to the nuclear wall (Fig. 1). In dividing secondary spermatogonial cells the mass of granules is arranged in irregular rows about the spindle, and fairly evenly divided without assuming any definite thread-like form. The granules visible in the fixed material can also be seen in the living cells. They are usually five or six in number, and do not stain with janus green. After being treated with neutral red for about an hour, however, they appear faintly pink. The number is often as large in the secondary as in the primary spermatogonia, and this gives some reason for supposing that

new granules of this sort are being gradually formed. While these granules cannot be followed during the spermatogonial divisions there seems little doubt that they lie inert in the cell and their distribution is merely hit or miss to one or the other of the daughter cells.

After the last spermatogonial telophase the nuclei enter on the stage shown in Fig. 2, in which the chromatin appears as a light network in prepared material. This network shortly becomes aggregated into flocculent masses corresponding roughly in number to the diploid number of the chromosomes. This "massive body" stage is beginning to appear in Fig. 3 and Fig. 4. The mitochondria in these early stages of the growth period are extremely difficult to demonstrate in fixed material—as indeed they are throughout the spermatogenesis—even by the special technique for mitochondria. At times, however, a cloudy mass can be seen forming a cap over one side of the nucleus (Fig. 2). With janus green, however, they become very prominent in the living material. The granules are larger and seemingly in greater numbers than in the spermatogonia. The details of their behavior are similar to those in *Chorthippus*, except that here again there is no tendency to assume the thread-like form.

Throughout these earlier stages of the growth period a mitosome, the remains of the last spermatogonial division, is present. Often an actual bridge between the two daughter cells persists for a short time after the division is complete. This is the condition seen in Fig. 3. The mitosome rapidly disappears after the stage shown in Fig. 3, and is never found after the "massive body" stage. At about this time there appears in about the center of the cytoplasmic mass a more or less definite sphere which is browned by osmic and only slightly stained by hæmatoxylin. At first I took this to be a mitosome, which it strongly resembles, but later it was found that a definite mitosome was present at the same time, as shown in Fig. 3. The sphere is shown with great clearness in Fig. 4, where it lies in a large vacuole—probably due to imperfect fixation—which is surrounded by masses of mitochondria. This sphere is not visible in the living cells, whether stained or not, but its presence can be inferred from the fact that in the growth period the mito-

chondria are usually in two separate masses with a clear space close to the nuclear wall between. An extreme case of this sort is shown in a cell in the bouquet stage (Fig. 6) which will be referred to later. Here it is very clear that a spherical mass must separate the mitochondria. This characteristic clumping of the mitochondria in two groups is mentioned by Lewis and Robertson, but no explanation is attempted. The behavior of this sphere shows that it is in the nature of an attraction sphere or idiozome (Meves).

Finally there are in the cytoplasm of these cells of the early growth period from three to six or more of the "neutral red granules" mentioned above. They are larger and more prominent than in the spermatogonia, and are shown stained with hæmatoxylin in Figs. 2 and 3. They may lie anywhere in the cytoplasm, but are more often among the mitochondria and close to the idiozome. In the living material they do not appear in the unstained cells, but when cells are stained with neutral red, they take the stain faintly some time after treatment. There are then four sorts of inclusions in the cytoplasm of the cells in the early stages of the growth period: (1) the mitosome, (2) the idiozome, (3) the fragments called "neutral red granules," (4) the masses of granular mitochondria.

From the stage of the massive bodies the chromosomes uncoil into the leptotene condition. The threads are fine and at first appear as a tangled mass. The monosome is of course an exception to this rule for it remains as a heavy densely staining mass. Shortly after the leptotenes begin to suggest a polarization, the threads become doubled and pass rapidly into the diplotene condition. Here they remain until the growth period is completed and diakinesis begins. In this process of polarization the spherical idiozome in the cytoplasm apparently plays an important part. It is not always visible in the stained material even when the preparations are carefully extracted with a view to making it clear, but in *every case* in which it can be seen in the late leptotene or diplotene stages it is found that the *chromatin threads are polarized towards it.* Two clear cases of polarized diplotenes are shown in Figs. 5 and 6. Both are approximately the same stage, the first from a fixed and mounted preparation,

and the second from a living cell stained with janus green. The first shows the diplotene threads clearly polarized toward the idiozome, while the second the indefinite heavy threads polarized toward a vacant space in the cytoplasm outlined by the mitochondria. After the diplotene stage the idiozome is never visible.

The failure of previous observers to identify an attraction sphere in at least some of the Acrididæ is rather peculiar, for it seems almost certain that it has been figured before. A body of similar appearance, called by Davis a "mitochondrion," is shown in at least his Figs. 31, 34 and 42. In view of the observed behavior of the mitochondria this is of course incorrect, and it seems improbable that it can be a mitosome at the stages indicated. As has been observed above, however, the idiozome is not always visible, and when taken in conjunction with the fact that a clear polarization of the threads is sometimes hard to find, this may indicate that the idiozome is a structure which is sometimes present and sometimes not.

It remains to trace the behavior of the "neutral red granules" during these earlier stages of the growth period. As stated above and shown in Figs. 2, 3 and 4, these granules are larger than the mitochondria, stain densely with hæmatoxylin, and may lie anywhere in the greatest mass of cytoplasm. When the diplotene stage is reached (cf. Fig. 5) we find instead of several granules a single rather large spherical mass lying in a clear vacuole. It is now exactly similar, though smaller, to the chromatoid body of *Pentatoma seilis* described by Wilson. This body stains very heavily with hæmatoxylin, while in the living cell it is practically invisible. When the living material has been treated with neutral red for about an hour, however, it appears as a highly refractive pink drop in the cytoplasm (Fig. 8). Intermediate stages between these two conditions have not been found in the living material, and even in the fixed preparations one usually finds that the cells from the massive body stage on, contain a single chromatoid body in the cytoplasm. Cells are found now and then in which several granules are apparently fused in one mass, or in which two seem in the process of fusion, as in Fig. 3. It is certain at least that the single chromatoid body appears at the expense of the earlier small granules, and this fact makes it

probable that they have become fused into one mass. Occasionally, however, cells appear in which a granule is present in addition to the larger chromatoid body, and I have observed one case in which two equal granules—or chromatoid bodies—were present in a cell at the time of diakinesis. The mass of the two would approximately equal that of the single body usually found at this time. It is possible therefore that the chromatoid body represents simply the enlargement of one of the smaller granules, as suggested by Wilson, who observed a few granules in the cytoplasm in the presynaptic stages of *Pentatoma*. In any case the single chromatoid body in *Rhomaleum* is undoubtedly made up of the material of the granules seen in the cytoplasm of the spermatogonia and early growth period.

Throughout the growth period the chromatoid body increases in size. When the early diakinesis is reached the cell has reached its maximum size, and at this point the chromatoid body is also largest. Fig. 7 shows a cell in which it was even larger than usual, while Fig. 8 represents a living cell at about the same stage, stained with both janus green and neutral red. The mitochondria at this stage are scattered about the cytoplasm to some extent, though they still show a tendency to be aggregated in a mass at one side of the nucleus. From this point onward the chromatoid body behaves as an inert mass, which does not increase in size, and is not affected in the slightest degree by either maturation division. At each division the mitochondria are arranged in the manner described by Lewis and Robertson, except that here again I noticed but little tendency to the thread-like form so evident in *Chorthippus*. The granules are arranged in rows about the spindle but their separate granular condition is always evident. The chromatoid body remains in whichever half of the cell the division plane happens to place it. It is sometimes within the mass of spindle fibers as in Fig. 9, which represents a first spermatocyte telpohase, but more often toward the periphery of the cell. Fig. 10 shows a very late telophase of the second maturation division. The nuclei have already assumed the flocculent appearance characteristic of the early spermatids. The cells are completely divided except for the spindle which still bridges them. The mitochondrial masses

have already become aggregated into the definite spheroidal nebenkerns, and the cytoplasm is clear and free from granules. The chromatoid body, here unusually small, is seen in one of the cells close to the wall of the nebenkern.

It is unnecessary to give details of the metamorphosis of the spermatids. In living material stained with janus green Lewis and Robertson have shown that the nebenkern appears as a mottled spherical mass, which finally divides into hemispheres, between which the axial filament passes. These granular hemispheres elongate as the tail draws out, eventually forming two dotted lines, one on either side of the axial filament. Here again the mitochondria appear as separate granules, with little tendency to fusion even in the mature sperm tail. In a few preparations stained by the Benda method I have noticed the "acrosome sphere," described by Meves and by Montgomery at the opposite side of a spermatid from the nebenkern (Fig. 11). It stains purple, which is the typical mitochondrial reaction. I have been unable to trace its origin or subsequent history, but by analogy with other forms it probably forms the perforatorium of the mature sperm. Since the chromatoid body never divides it should be found in one fourth of the spermatids. In order to test this expectation ten cysts of spermatids in various stages were selected at random from several preparations, and the total number of cells and chromatoid bodies recorded. The results follow:

No. of Cyst.	No. of Spermatid Nuclei.	No. of Chromatoid Nuclei.
1	59	12
2	78	21
3	56	15
4	54	11
5	64	13
6	40	9
7	50	11
8	101	22
9	61	15
10	56	14
Total	619	143

The 143 chromatoid bodies observed are fairly close to the expected number 155.

With the elongation of the spermatid the chromatoid body

wanders further and further from the nucleus, and usually lies close to the axial filament (Fig. 12). It migrates eventually to a considerable distance down the tail, where it may be seen, still in its vacuole, forming a swelling at one side of the axial filament in the nearly mature sperms (Fig. 13). When the metamorphosis is complete no bodies are seen in the tails themselves, but scattered among them are numerous deeply staining bodies of the same size in various stages of degeneration. This condition is plainly visible in the living material where the loose granules stain quickly and brilliantly with neutral red. The process is therefore identical with that in *Pentatoma*, except that it is unusual to find any great amount of protoplasm cast off from the sperm tails with the chromatoid bodies.

Staining Reactions of the Chromatoid Body.

As has been noted above the chromatoid body in *Rhomaleum* gives the specific reaction when the living cells are treated with neutral red which has been described for certain granules of similar behavior in *Chorthippus*. Lewis and Robertson have also observed that somatic and apical cells often appear to be crowded with material which gives a similar though more distinct relation. I am able to confirm their observation with regard to the apical cell in *Rhomaleum*. It appears, therefore, that a specific substance which is an ordinary inclusion of the cytoplasm of certain somatic cells and of the apical cell is present in the cytoplasm of the spermatogonial and early growth period cells of some grasshoppers in small quantities, that it increases in amount during the growth period, and may become aggregated into one mass. Like almost any foreign substance in the cytoplasm this mass appears to lie in a vacuole in fixed material. It is finally eliminated from the nearly mature spermatozoa. That a similar condition occurs in the male germ-cells of many other animals is made probable by the fact that similar bodies have been described in an increasing number of forms.

As to what this substance is, one can give no certain answer. In the living cells it remains almost invisible when unstained, and it appears faintly pink after a rather long treatment with neutral red. In the fixed material the body stains densely with

hæmatoxylin, safranin and other chromatin stains. When the Flemming triple combination is used the chromatoid body is red throughout, even though the resting nuclei are purple. With the Auerbach stain the body is clearly differentiated from the chromatin, for it is bright red while the nuclei are green. With the Benda alizarin-crystal violet method, even when the material is fixed as directed with the modified Flemming's fluid, the mitochondria seldom appear, and the nuclei and chromatoid body appear bright purple. I have tried this method repeatedly with the germ cells of *Rhomaleum* but have never been able to get the brilliant result shown by Giglio-Tos and Granata (1908) in their paper on *Pamphagus marmoratus*. With the Altmann acid fuchsin method the chromatoid body is clear red as are the mitochondria. The material is therefore unlike either mitochondria or chromatin in chemical constitution, a fact clearly shown by its behavior.

I am glad of an opportunity to express my indebtedness to Professor E. B. Wilson for generous advice in the preparation of this paper as well as for numerous suggestions with regard to technical methods. I am also indebted to Miss Mabel Hedge for the original drawings of Figs. 3 and 4.

SUMMARY.

The mitochondria in *Rhomaleum* are shown to be present in the spermatogonia. Their behavior agrees closely with that described by Lewis and Robertson for *Chorthippus*, except that they remain granular throughout.

There are present in the spermatogonial cells of *Rhomaleum* in addition to the mitochondria certain fine granules which stain in contrast to the mitochondria with neutral red. These granules are carried over into the early spermatocytes where they probably become aggregated into one mass. This mass grows for a short period, passes inert through the two maturation divisions, and into one fourth of the spermatids. From the tails of the developing sperm it is cast off into the end of the follicle, where it degenerates. In addition it has been noted that an idiozome or attraction sphere is present in the early spermatocytes of *Rhomaleum*, and an acrosome sphere in the spermatids.

COLUMBIA UNIVERSITY,
October 5, 1916.

LITERATURE CITED.

Meves, F.

'00 Ueber den von v. la Valette St. George entdeckten Nebenkern. **Arch.** Mickr. Anat., Bd. 56.

Davis, H. S.

'08 Spermatogenesis in Acrididæ and Locustidæ. Bull. Mus. Comp. Zool. Harvard Univ., Vol. 53, No. 2.

Giglio-Tos, E., and Granata, L.

'08 I., Mitocondri nelli cellule seminali maschili di Pamphagus marmoratus. Biologica, V. II., No. 4.

Wilson, E. B.

'13 A Chromatoid Body in Pentatoma. BIOL. BULL., May, 1913.

Lewis, M. R., and Robertson, W. R. B.

'16 Mitochondria and other Structures in Chorthippus. BIOL. BULL., Feb., 1916.

Explanation of Plate I.

All figures except Nos. 1, 6 and 8 from camera drawings made with Leitz 1.5 mm. oil immersion objective and 8 compensating ocular. Nos. 1, 6 and 8 from living material with 2 mm. objective.

Fig. 1. Resting stage of secondary spermatogonia—living cell stained with janus green showing granular mitochondria.

Fig. 2. First spermatocyte showing chromatin network with cloudy mass of mitochondria and prominent "neutral red granules" in the cytoplasm.

Fig. 3. First spermatocyte beginning to show the "massive bodies"; mitosome, idiozone and "neutral red granules" visible in cytoplasm.

Fig. 4. Similar to the preceding, the idiozome shrunken so as to appear in a vacuole because of imperfect fixation.

Fig. 5. First spermatocyte bouquet stage—diplotene threads polarized toward idiozome, chromatoid body visible.

Fig. 6. Similar to preceding—living cell stained with janus green—mitochondria in two masses surrounding a spherical space in which the idiozome (invisible) probably lies, since the diplotene threads are polarized toward it.

Fig. 7. Diakinesis—drawn from a preparation stained with alizarin and crystal violet—showing large chromatoid body in a vacuole.

Fig. 8. Similar stage in living cell stained with both janus green and neutral red,—mitochondria and chromatoid body visible.

Fig. 9. Telophase of first spermatocyte division—the chromatoid body passing to one pole.

Fig. 10. Late telophase of second spermatocyte division—spermatid nuclei already formed—mitochondria aggregated into the spherical nebenkerns-chromatoid body in one daughter-cell.

Fig. 11. Spermatid showing nebenkern and acrosome sphere.

Fig. 12. Spermatid, tail beginning to elongate, nebenkern divided, chromatoid body passing down close to the axial filament.

Fig. 13. Group of sperm tails, showing chromatoid bodies just before they are cast off.

FIG. 1.

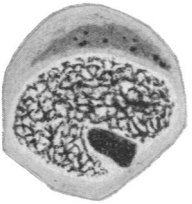

FIG. 2.

FIG. 3.

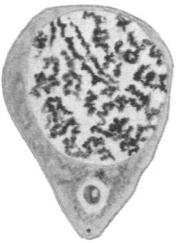

FIG. 4.

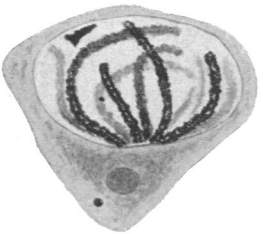

FIG. 5.

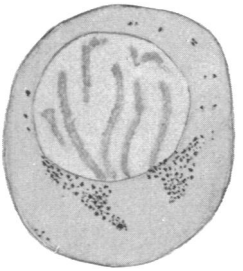

FIG. 6.

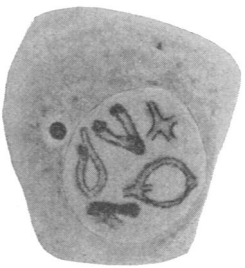

FIG. 7

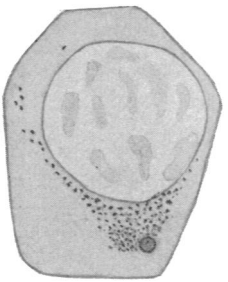

FIG. 8.

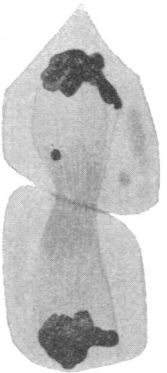

FIG. 9.

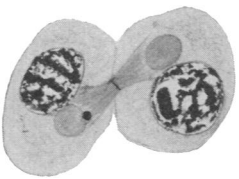

FIG. 10.

FIG. 11.

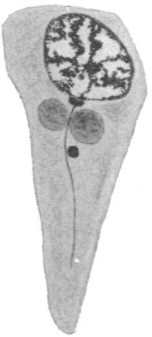

FIG. 12.

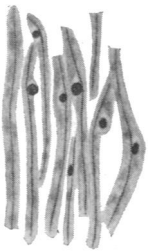

FIG. 13.

HAROLD H. PLOUGH.